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Search for Human Herpesvirus 6 and Human Cytomegalovirus in Bronchoalveolar Lavage From Patients With Human Immunodeficiency Virus-1 and Respiratory Disorders

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Virus isolation and viral DNA detection by the polymerase chain reaction were used to investigate the presence of human herpesvirus 6 (HHV-6) and human cytomegalovirus (HCMV) in bronchoalveolar lavage from 34 human immunodeficiency virus-1 (HIV-1)-infected patients with respiratory disorders. The aim was to assess the presence of reactivated HHV-6 in lung tissues for a subsequent evaluation of the frequency of virus involvement in respiratory clinical manifestations in the course of HIV-1 infection. Bronchoalveolar lavage samples were tested for the presence of HCMV, as a routine investigation within a protocol monitoring opportunistic infections in symptomatic HIV-1 patients. Whereas HCMV DNA was detected by the polymerase chain reaction in 12 bronchoalveolar lavage specimens, 10 of which were also positive for virus isolation, all samples were negative for HHV-6 by both virological procedures. The HHV-6 DNA finding in bronchoalveolar lavage from an HIV-1-seronegative patient with renal carcinoma, investigated accidentally together with the bronchoalveolar lavage specimens from HIV-1 seropositive patients, stressed the HHV-6 polymerase chain reaction-negative results in the bronchoalveolar lavage samples under study. It is concluded that the lung may be a target organ for HCMV infection in HIV-1-seropositive patients affected by respiratory symptoms but that this does not seem to be the case for HHV-6.

KEY WORDS: human herpesvirus 6, human cytomegalovirus, human immunodeficiency virus-1, bronchoalveolar lavage, pulmonary disease

INTRODUCTION

Human herpesvirus 6 (HHV-6) is widespread in humans as shown by the 80–90% antibody frequency in the adult population [Saxinger et al., 1988; Okuno et al., 1989; Cermelli et al., 1992]. HHV-6 primary infection, occurring usually in the first 2 years of life [Briggs et al., 1988], may cause exanthem subitum [Yamanishi et al., 1988] or a febrile illness without rash [Portolani et al., 1990]. In addition, HHV-6 has been associated with a number of different clinical manifestations including mononucleosis-like conditions [Hanukoglu and Somekh, 1994], benign and malignant lymphoproliferative disorders [Harrett et al., 1988; Torelli et al., 1991; Krueger et al., 1992], chronic fatigue syndrome [Josephs et al., 1991], fatal haemophagocytic syndrome [Huang et al., 1990], and pneumonitis in bone marrow transplant recipients [Carrigan et al., 1991; Cone et al., 1993]. After primary infection, HHV-6 may persist in the host over a life-time and immunosuppression may trigger the rescue of latent virus [Drobyski et al., 1993]. HHV-6 antigens have been detected in autopsy tissues from many organs in subjects who died from human immunodeficiency virus-1 (HIV-1) infections [Corbellino et al., 1993; Knox and Carrigan, 1994] and a high frequency of HHV-6 has been implicated as the cause of interstitial pneumonitis in the course of HIV-1 infection [Knox and Carrigan, 1994]. It was therefore decided to assess the presence of HHV-6 in bronchoalveolar lavage from HIV-1-infected patients with respiratory disorders. The bronchoalveolar lavage samples were sent to the laboratory from the Division of Infectious Diseases of the University Hospital for routine human cytomegalovirus (HCMV) investigation monitoring opportunistic infections in HIV-1 symptomatic patients.

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MATERIALS AND METHODS

Patients

Bronchoalveolar lavage from a cohort of 34 HIV-1-seropositive patients with a CD4+ T lymphocyte percentage varying between a 15–4% range and bronchoalveolar lavage from an HIV-1-seronegative patient immunocompromised by renal carcinoma accidentally included among bronchoalveolar lavage samples of HIV-1 patients were examined. Bronchoalveolar lavage was undertaken for respiratory symptoms characterised by cough, dyspnoea, and fever.

Serological Studies

IgG antibodies to HCMV were investigated in serum samples of the patients under study with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Behringwerke, Marburg, Germany) and to HHV-6 with an indirect immunofluorescence assay (IFA) which employed cultures of cord blood lymphocytes infected with an HHV-6 isolate obtained in our laboratory from a child with exanthem subitum [Portolani et al., 1990].

Virological Studies

The presence of HCMV and HHV-6 in bronchoalveolar lavage samples was investigated both by virus isolation and viral DNA detection by polymerase chain reaction (PCR). Firstly, 2 ml from each bronchoalveolar lavage sample supplemented with antibiotics were clarified by centrifugation at 2,000 rpm for 20 minutes. Human fibroblasts grown in culture tubes and shell vials and cord blood lymphocyte cultures were inoculated with a volume of 0.2 ml of the bronchoalveolar lavage supernatant for the isolation of HCMV and HHV-6, respectively. Human fibroblast cultures in shell vials were monitored for HCMV p76 antigen with a monoclonal antibody (Clonatec, Paris, France) in an IFA, 12–24 hours after culture inoculation. HCMV and HHV-6 growth was monitored in the respective cell cultures for the presence of viral cytopathic effect over a period of 3 weeks. The remaining amount of each bronchoalveolar lavage sample was centrifuged at 1,800 rpm for 10 minutes. The cell sediment was counted in a Burker camera and then frozen at −20°C until processed by PCR.

PCR and Nested PCR Assays

The cell sediment from each bronchoalveolar lavage was resuspended in an appropriate volume of lysis buffer to obtain a concentration of 10^6 cells/100 μl. The lysis buffer contained Tris-HCl (pH 8) 50 mM, NaCl 70 mM, Triton X-100 0.0001%, SDS 0.00001%, and proteinase K 0.2 mg/ml. The samples were incubated at 56°C for 1 hour and then proteinase K was inactivated at 95°C for 10 minutes. For each PCR assay an amount of cell lysate corresponding to 2×10^5 cells was diluted in 50 μl of an amplification mixture containing KCl 50 mM, Tris-HCl (pH 8.3) 10 mM, $MgCl_2$ 1.5 mM, the four dNTPs each at a concentration of 200 μM, 50 pm of each primer, and 1.2 U Taq polymerase. In order to ascertain the amplificability of the DNA obtained and to avoid false-negative results, the samples were amplified with a pair of primers (BG3 and BG4) for human β-globin gene before assaying with CMV and HHV-6 primers.

Primers p5 and p6 [Rawal et al., 1994] were used to detect HCMV DNA in the samples: this pair of primers amplifies a fragment of 234 base pairs (bp). The amplification was carried out for 40 cycles in a thermal cycler (MJ Genenco, Florence, Italy) according to the following programme: initial denaturation at 96°C for 5 minutes, annealing at 57°C for 30 seconds, extension at 72°C for 90 seconds, and denaturation at 94°C for 30 seconds; final extension was carried out at 72°C for 10 minutes. After electrophoresis migration in a 3% agarose gel, the amplification product was visualized by ethidium bromide staining. Positive and uncertain samples were processed with a nested (n) PCR to confirm the PCR results. In this case, for the internal amplification, a pair of primers [p7 and p8, Rawal et al., 1994] which amplify a fragment of DNA of 168 bp internal to that amplified by p5 and p6, was employed. The amplification was carried out for 30 cycles as described above.

Primers A and C, yielding a product of 830 bp, were used to detect HHV-6 DNA in the samples, according to Aubin et al. [1991]. The amplification programme consisted of an initial denaturation at 92°C for 7 minutes, 40 cycles with an annealing step at 55°C for 1 minute, an extension step at 72°C for 1 minute 25 seconds, and a denaturation step at 92°C for 1 minute, final extension increased to 7 minutes. The positive or uncertain samples were then amplified with primers HS6AE and HS6AF [Dewhurst et al., 1993] internal to A and C, in a 30 cycle programme consisting of an initial denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, extension at 72°C for 1 minute (with an increase of 12 seconds at each cycle), denaturation at 94° for 1 minute, final extension prolonged for 10 minutes.

Restriction Endonuclease Analysis

Viral DNA from the HHV-6-positive bronchoalveolar lavage sample was characterized by restriction endonuclease analysis [Di Luca et al., 1992]. PCR products obtained with primers A and C were digested by two restriction enzymes (Hind*III* and Hin*fI*) for 2 hours at 37°C and then the restriction patterns visualized by ethidium bromide staining, after electrophoresis migration on a 2% agarose gel.

RESULTS

Table I illustrates the presence of HCMV and HHV-6 in bronchoalveolar lavage of the 34 HIV-1-positive patients as assessed by virus isolation and by PCR technique; the Table also shows the results of serological tests against both viruses. IgG specific for HCMV were detected in the serum of 29 patients (85.3%), whereas IgG for HHV-6 were revealed in 32 (94.1%). An HIV-1-seronegative patient immunocompromised by renal carcinoma was found seropositive to both HCMV and

TABLE I. HCMV and HHV-6 Serological and Virological Results*

Patient no.	Abs to		Viral isolation		PCR[a]		nPCR		Aetiology of the illness
	HCMV	HHV-6	HCMV	HHV-6	HCMV	HHV-6	HCMV	HHV-6	
1	+	+	–	–	–	–			Bacterial
2	+	+	+	–	+	–			Pneumocystis
3	+	+	+	–	+	–	+		Pneumocystis
4	+	+	–	–	–	–			Bacterial
5	+	+	+	–	+	–			Cryptococcus
6	+	+	–	–	–	–			Cryptococcus
7	+	+	–	–	+	+	–	–	Pneumocystis
8	+	+	–	–	–	–			Unknown
9	+	+	+	–	+	–			Bacterial (TBC)
10	–	+	–	–	–	–			Bacterial
11	–	+	–	–	–	–			Pneumocystis
12	–	+	–	–	–	–			Bacterial
13	+	+	–	–	–	+		–	Unknown
14	–	+	–	–	–	+		–	Pneumocystis
15	+	+	+	–	+	–			Unknown
16	+	+	–	–	+	–	+		Unknown
17	+	+	–	–	–	–			Bacterial
18	+	+	–	–	–	–			Bacterial
19	+	+	+	–	+	–			Unknown
20	+	–	–	–	+	–	+		Pneumocystis
21	+	+	+	–	+	–			Pneumocystis
22	+	–	+	–	+	–			Unknown
23	–	+	–	–	–	–			Pneumocystis
24	+	+	–	–	–	–			Bacterial
25	+	+	–	–	–	–			Bacterial
26	+	+	+	–	+	–	+		Pneumocystis
27	+	+	–	–	–	–			Unknown
28	+	+	–	–	–	–			Bacterial
29	+	+	–	–	–	–			Bacterial
30	+	+	–	–	–	–			Unknown
31	+	+	–	–	–	–			Bacterial
32	+	+	–	–	–	–			Unknown
33	+	+	+	–	+	–	+		Pneumocystis
34	+	+	–	–	–	–			Unknown

*Microbiological diagnosis of the respiratory disorders.
[a]PCR results not confirmed by nPCR were considered negative.

HHV-6. HCMV isolation was positive in bronchoalveolar lavage from 10 of the 29 HCMV seroimmune subjects (34.5%). With PCR assay, three additional bronchoalveolar lavage samples were found positive but the HCMV-DNA presence was confirmed by nPCR in only two of these specimens (Fig. 1) out of a total of 12 HCMV-positive bronchoalveolar lavage samples (41.4%). Attempts to isolate HHV-6 from bronchoalveolar lavage samples were negative; the PCR positivity of three samples was not confirmed by nPCR (Table I). HHV-6 DNA was found in the bronchoalveolar lavage drawn from the HIV-1-seronegative patient immunocompromised by renal neoplasm. A strongly positive signal was still detected when a dilution of the cell lysate sample corresponding to DNA from 1.2×10^4 cells was processed by PCR. As it appears in Figure 2, the viral DNA, analyzed with restriction endonucleases, shows a profile corresponding to that of HHV-6 group A DNA.

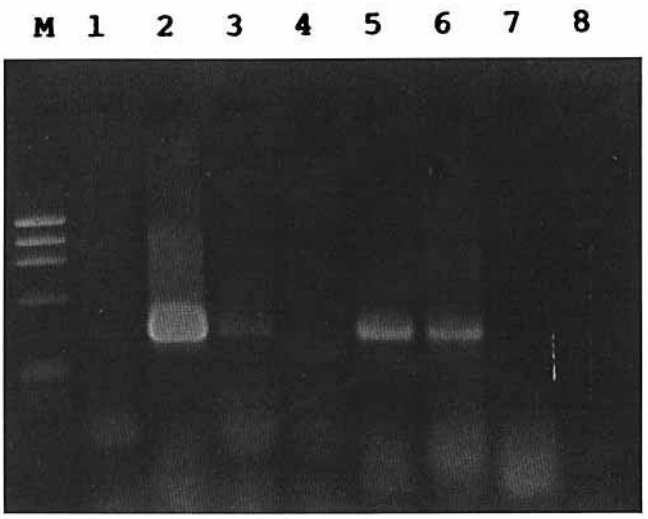

Fig. 1. Ethidium bromide staining of PCR products obtained from bronchoalveolar lavage samples assessed for HCMV-DNA. **M**, molecular weight marker (digested Φ DNA); DNA extracted from uninfected (**lane 1**) and HCMV-infected cells (**lane 2**); PCR weakly positive bronchoalveolar lavage sample (**lane 3**) not confirmed by nPCR (**lane 4**); PCR-positive (**lanes 5, 6**) and PCR-negative bronchoalveolar lavage samples (**lanes 7, 8**).

DISCUSSION

The presence of HCMV and HHV-6 was investigated in bronchoalveolar lavage collected from 34 HIV-1 patients with respiratory disorders. All bronchoalveolar

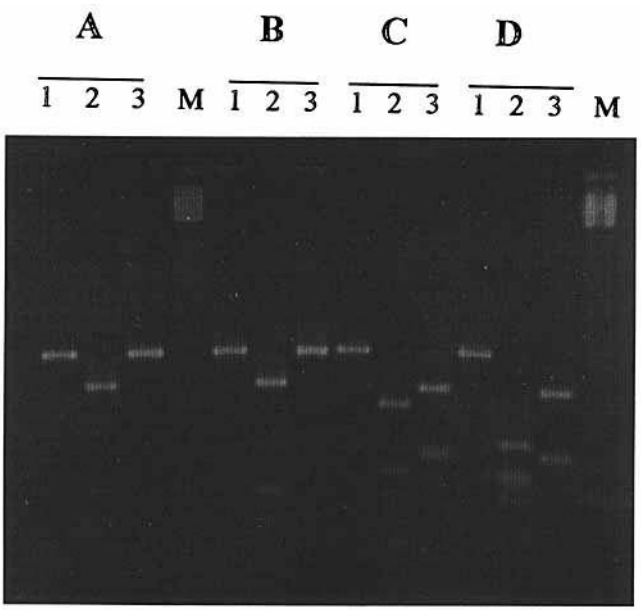

Fig. 2. Characterization of PCR-amplified HHV-6 DNA from bronchoalveolar lavage of the HIV-1-seronegative patient. **M,** molecular weight marker (123 bp ladder). Pattern of PCR-amplified HHV-6 DNA from patient's bronchoalveolar lavage **(lane A).** HHV-6 DNA pattern of strain U1102, group A **(lane B);** strain Z29, group B1 **(lane C);** and strain CV, group B2 **(lane D).** DNA samples undigested **(lanes 1),** digested by Hinf*I* **(lanes 2),** and Hind*III* **(lanes 3).**

lavage samples, 32 of which were from HHV-6-seroimmune patients, were negative for the presence of HHV-6 when analyzed both by viral isolation and PCR technique. The presence of HCMV-DNA was detected in bronchoalveolar lavage from 12 of 29 HCMV-immune patients (41.4%). These HCMV-DNA findings correlated with the HCMV isolation results which were positive in 10 of the 12 PCR-positive samples (83.3% of concordance). The sensitivity of the virological procedures to detect the presence of HHV-6 was comparable to those used for HCMV detection, the PCR detection limit for both target DNAs being 4×10^4 molecules [Rawal et al., 1994; Di Luca et al., 1994]. Thus, the absence of HHV-6 in bronchoalveolar lavage specimens cannot be attributed to deficiencies in the investigation techniques. Also the finding of HHV-6 DNA in the bronchoalveolar lavage sample from the only HIV-1-seronegative patient ruled out technical reasons for the negative results. The fact that HHV-6, unlike HCMV, shares with HIV-1 CD4+ T lymphocytes as cell targets could account for the different results on the finding of the two viruses. Recently, two groups of investigators have shown independently that in symptomatic patients, high loads of HIV-1 virions are produced by newly infected lymphocytes and cleared rapidly [Ho et al., 1995; Wei et al., 1995]. Competition with a large population of HIV-1 particles to infect permissive cells and/or the viral but chiefly the immune killing of HIV-1-infected cells which probably underlies the virus clearance could hamper the expansion of occasionally rescued HHV-6 in the lungs. This would result either in the absence of HHV-6 virions or such a dispersion in the bronchoalveolar lavage to make the virus detection unlikely even with PCR assays.

A high viral load is not required to cause an organ-specific pathology. Nevertheless, the PCR negativity for HHV-6 of bronchoalveolar lavages from a representative cohort of HIV-1-seropositive patients with respiratory disorders probably associated with a picture of interstitial pneumonitis in several patients, contrasts with the hypothesis of HHV-6 as a frequent opportunistic agent of respiratory illness in the course of HIV-1 infection. HHV-6 could cooperate in the HIV pathogenesis by both the enhancement of HIV-1 replication and depletion of CD4+ T lymphocytes [Lusso et al., 1989] rather than by the injury of a specific organ target. This latter event could occur occasionally during HIV-1 infection as well as in other immunosuppressive circumstances. An example was observed in this study. HHV-6 detected by PCR in bronchoalveolar lavage from the HIV-1-uninfected subject with a renal carcinoma was probably responsible for the lymphocyte pneumonitis diagnosed in the patient since no finding of other infectious agents, HCMV included, was possible. It is of interest that the virus belonged to HHV-6 group A, less frequently found than group B.

When the patients' charts were examined, the results of microbiological studies carried out at the same time as the investigations showed that of the 34 cases of respiratory disorders, 12 were of bacterial origin, 10 correlated with pneumocystis carinii, 2 with cryptococcus neoformans, and 10 were of unknown aetiology (Table I). HCMV was isolated from seven of nine HCMV-seroimmune patients with pulmonary disease due to pneumocystis or cryptococcus vs. only 1 of 10 HCMV-seroimmune patients with pulmonary infection of bacterial origin (77.8 vs. 10%, $P = 0.000$ for the chi-square test with Fisher's correction). The cell-mediated immune response is known to be important for the control of eukaryotic infections and vice versa for most infections of prokaryotic aetiology. The significantly higher frequency of HCMV rescue associated with respiratory diseases by pneumocystis and cryptococcus suggests that HCMV reactivation is correlated with peculiar cell-mediated events rather than with humoral deficiencies. That the only case of bacterial respiratory infection associated with the HCMV rescue was a case of tubercular pneumonitis (patient 9) confirms this supposition as well as the fact that HCMV IgG antibodies at significantly higher titres were present in patients with respiratory infections by eukaryotic than prokaryotic microorganisms (mean optical density values: 1.62 vs. 1.06, respectively, $P = 0.031$.). Of the 10 patients with microbiologically negative bronchoalveolar lavage, 4 (15, 16, 19, 22) had bronchoalveolar lavage positive for HCMV. In the absence of any other documented infectious agent, the pathogenetic role of HCMV cannot be ruled out especially in two of these patients (15 and 19) who presented HCMV-positive antigenaemia and viraemia in addition to HCMV in bronchoalveolar lavage. Thus, in the course of HIV infection, the lungs as

well as the retina, the peripheral and the central nervous system, and gastrointestinal tract may represent a target organ for HCMV infection.

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